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Microvesication in human skin, due to ex vivo exposure to HD, can be blocked by 1) hydroxamate-based inhibitors of MMPs and related enzymes, 2) furin inhibitor and 3) pancaspase inhibitor. The observation that one of the hydroxamate-based inhibitors, BB94, can be applied until 18h after exposure to HD suggests that non-urgent cure of HD casualties is possible.

During pathogenesis of HD-induced microvesication the expression of laminin-5, one of the key proteins in epidermal dermal attachment, is reduced. However, although laminin-5 is target protein for MMps and related enzymes, these enzymes are not responsible for the decrease in laminin-5 expression. Moreover, epidermal-dermal attachment is still possible, in spite of reduced amounts of laminin-5. Cleavage of other attachment proteins than laminin-5 by MMps and related enzymes probably causes the break between epidermis and dermis. Impediment of the apoptotic cell death process appears to be also very effective in maintaining epidermal-dermal adhesion. The observation that caspase inhibitors prevent fragmentation of keratins 14, 16, and 17 in HD-exposed HEK may suggest that an intact cytoskeleton of keratin filaments is essential for epidermal-dermal adhesion. The observation that caspase inhibitors prevent fragmentation of keratins 14, 16, and 17 in HD-exposed HEK may suggest that an intact cytoskeleton of keratin filaments is essential for epidermal-dermal attachment.

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List of abbreviations

ADAM	A disintegrin and metalloprotease
AEC	3-amino-9-ethylcarbazol
BM	Basement membrane
CHAPS	3-[3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
DNase	Deoxyribonuclease
DTT	1,4-Dithiotreitol
HD	Sulfur mustard
HRP	Horseradishperoxidase
HSP	Heat shock protein
HEK	Human epidermal keratinocytes
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
kDa	Kilodalton
KGM	Keratinocyte growth medium
KBM	Keratinocyte basal medium
MALDI-TOF	Matrix- assisted laser desorption/ionization – time of flight
µM	Micromolar
mM	Millimolar
MMP	Matrix metalloproteinase
MS	Mass spectrometry
MW	Molecular weight
PBS	Phosphate buffered saline
pI	Isoelectric point
RC/DC	Reductant compatible/detergent compatible
RNase	Ribonuclease
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TACE	Tumor necrosis factor-alpha converting enzyme
Tris	Tris(hydroxymethyl)-amino-methane

1 Introduction

The studies described in this report are part of a research project (award number DAMD17-02-1-0206) aimed to obtain adequate medical defense against injury caused by exposure to sulfur mustard (HD). The project will elucidate the mechanism behind epidermal-dermal separation in HD-exposed human skin in order to bring a causal therapy against blister formation. Loss of the attachment competence of the basement membrane (BM) proteins is postulated to be a specific cause of HD-induced vesication of the skin and a disturbed balance between production and degradation of these proteins has been suggested contributory. Hence, in order to prevent microvesication, degradation of BM proteins should be diminished and/or their production should be enhanced.

It is known that BM proteins are specifically degraded by matrix metalloproteinases (MMPs), a family of enzymes that is involved in normal turnover of the extracellular matrix (Kleiner and Stetler-Stevenson, 1999; Nagase and Woessner, 1999). In the present study, various inhibitors of MMPs were tested on their potential to reduce HD-induced microvesication by preservation of BM proteins. On the other hand, maintenance of BM proteins can also be achieved by ensuring their synthesis. HD-induced epidermal cell death will cause a hold up of protein synthesis. Based on indications that apoptosis is involved in HD-induced cell injury (Rosenthal et al., 2003; own observations), it was tested whether block of apoptosis by caspase inhibition would have beneficial effects on the maintenance of the BM.

Laminin-5 is a key protein in epidermal-dermal attachment, and it has been described that the protein is substrate for MMPs and related enzymes (Gianelli et al., 1997; Pirila et al., 2003; Veitch et al., 2003). To visualize changes in the expression of laminin-5 following HD exposure and the possible effects of inhibition of MMPs and caspases, immunohistochemical staining has been used.

Finally, we analyzed the protein patterns of control and HD-treated cultured human epidermal keratinocytes (HEK) as a global approach to reveal cellular events occurring after HD exposure. In the protein profile of HD-exposed HEK, many fragments of keratins 14, 16 and 17 were observed. Since fragmentation of cytoskeletal proteins is related to apoptosis, it was tested whether formation of these fragments could be prevented with inhibitors of apoptosis.

2 Experimental Methods

2.1 HD synthesis

HD has been synthesized at TNO-Prins Maurits Laboratory and has a purity of at least 97%.

2.2 Exposure of skin pieces to HD vapor and human skin organ culture

Human mammary skin was obtained from cosmetic surgery with informed consent of the patient.

Human mammary skin has been exposed to saturated HD vapor at 25 °C using a vapor cup device as described earlier (Mol et al., 1991). The duration of exposure to HD vapor was five minutes, unless otherwise indicated. Organ cultures of human skin were maintained as described by Varani et al. (1995). Skin pieces of 0.25 cm^2 were floated with the

dermal side down in keratinocyte basal medium (BioWhittaker Europe, Verviers, Belgium) supplemented with $CaCl_2$ to a final concentration of 1.4 mM (KBMCa; 1 ml medium/well of a 12 well cluster plate) and incubated at 37 °C in an atmosphere of 6% CO_2 in air. Post-exposure incubation was for 48 h, unless otherwise indicated.

The MMP inhibitor BB94 (a gift of British Biotech, Oxford, UK), the pancaspase inhibitor Z-VAD- FMK (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands), the MMP-2/MMP-9 inhibitor (Calbiochem, San Diego, USA), inhibitor of MMP-1, -3, -7 and -9 (Inhibitor II, Calbiochem, San Diego, USA) and inhibitor of MMP-1, -2, -3, -7, -13 (Inhibitor III, Calbiochem, San Diego, USA) were dissolved in DMSO and diluted in organ culture medium to the desired final concentrations. The final concentration of DMSO was always 1%. The furin inhibitor Dec-RVKR-CMK (Bachem AG, Bubendorf, Switzerland) was directly dissolved in organ culture medium to the desired final concentrations.

2.3 Histology

Human skin pieces were fixed overnight at 4 °C in 2% paraformaldehyde in phosphate buffered saline (PBS). They were stored in 70% ethanol until embedding in paraffin. Sections were stained with hematoxylin/eosin and examined by light microscope. Photographs were taken by a digital camera (Olympus C5050 Zoom).

2.4 Immunohistochemistry

Human skin pieces were frozen in Tissue-Tek OCT embedding compound and 5 μ cryostat sections were cut. To prevent degradation of laminin-5, slides with cryosections were frozen immediately after cutting and kept frozen until further processing. After blocking with non-specific proteins, sections were incubated overnight at 4°C with monoclonal mouse anti-human laminin-5 (clone GB3), followed by 1 h incubation at room temperature with biotinylated goat anti-mouse IgG. The immunoperoxidase staining was perfomed using an avidin- horseradish-peroxidase (HRP) conjugate and 3-amino-9-ethylcarbazol (AEC). Slides were counterstained with haematoxylin and mounted under a coverslip. Negative controls were performed by omitting the primary antibody during the first incubation. Photographs were taken by a digital camera (Olympus C5050 Zoom).

2.5 Human epidermal keratinocyte (HEK) culture and exposure of HEK to HD

First passage HEK was grown confluent in serum-free keratinocyte growth medium (KGM) (BioWhittaker Europe, Verviers, Belgium). To study the cellular response of the cells to HD, the cells were exposed for 30 min to 0, 100 or 150 M HD.

One day after reaching confluence, HEK were exposed to HD (2.5 ml/well of a 6-well cluster plate) for 30 min at 25 °C. Stock solutions of HD were freshly prepared in dry acetone and diluted immediately before use in KBM to obtain the desired working concentrations. The final concentration of acetone in the incubation medium was 1%.

Following 2, 4, 8, 18, 24, 32 and 42 h of post-exposure incubation with fresh KGM the cultures were prepared for two dimensional polyacrylamide gelectrophoresis (2D-PAGE). Lysates of three wells with identical treatment were pooled.

To verify whether protein fragments were due to cleavage by caspases, HEK cultures were incubated with KGM containing 20 μM of the caspase-3 and -7 inhibitor Z-DEVD-FMK, or 20 μM of the caspase-6 inhibitor Z-VEID-FMK, or 20 μM of the pancaspase inhibitor Z-VAD-FMK (all caspase inhibitors were from Sigma Aldrich Chemie Zwijndrecht, The Netherlands). The caspase inhibitors were added 3 h prior the HD exposure and during the 24 h post-exposure period.

2.6 2D-PAGE

Cells were solubilized in 40 mM Tris buffer (pH 8.0) containing 0.5% SDS, and the lysate was boiled immediately for 10 min at 95°C. After cooling on ice, the lysates were incubated for 2 h at 4°C with 100 mM DTT, protease inhibitor cocktail (Complete ® Mini, Roche Diagnostics, Mannheim, Germany) and DNase I/ RNaseA (Roche Diagnostics, Mannheim, Germany). After sonification by four bursts of 5 s, proteins were precipitated in 11% trichloroacetic acid in ice-cold acetone supplemented with 25 mM DTT. Pellets were washed twice with ice-cold acetone and subsequently dissolved in 9.5 M urea, 4% CHAPS, 0.5% IPG buffer and 25 mM DTT. Protein content was determined with the RC/DC protein assay (BioRad). Samples of 150 µg protein were loaded on 13 cm Immobiline IPG strips (4-7) (Amersham Bioscience Biotech, Roosendaal, the Netherlands). Isoelectric focusing was carried out at 20° C to 33.8 kVh on an IPGphor apparatus. Before SDS-PAGE , the focused IPG strips were equilibrated, reduced and alkylated in a buffer containing 6 M urea, 50 mM Tris-HCl, pH 8.8, 2% SDS, 30 % glycerol with either 10 mg DTT/ml or 25 mg iodoacetamide/ml. Then the proteins were separated in the second dimension on homogeneous polyacrylamide gels (10% T). The SDS-PAGE was run at a constant current of 30 mA/gel at 20°C. Gels were stained by a mass spectrometry compatible silver staining (Yan et al., 2000). Comparative analysis of protein patterns has been performed partially by eye and partially by using ImageMaster 2D Elite software (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). For each comparison at least four replicate gels of each sample were used. For changes in proteins spots to be considered as genuine differences in protein expression we applied the following criterion: they should show similar qualitative changes in expression in all examined gels originating from one sample.

2.7 Protein identification

Since the percentage of successfully identified spots in our laboratory occurred to become relatively low, excised gel spots were send to BioCat (Heidelberg, Germany) for mass spectrometric identification. However, their yield of identified proteins was not better than ours. Therefore, we have optimized our previously used procedure for protein identification, specifically with regard to the trypsin source and the protein : trypsin ratio.

Spots of interest were excised and cut into 1-2 mm3 pieces. The particles were washed and dehydrated before they were digested with 100 ng trypsine sequencing grade (Promega, Leiden, The Netherlands) in 25 mM NH4HCO3 overnight at 37 °C on a shaker (Jensen et al., 1999). The peptides were extracted twice with 5% formic acid and acetonitrile (1:1) and the extracts were pooled and dried in a vacuum centrifuge. Before desalting the peptides were dissolved in 5% formic acid. Desalting was performed with ZipTip microcolumns of C18 (Millipore, Amsterdam Zuid-Oost, The Netherlands). Peptide mass fingerprints were determined by matrix assisted laser desorption ionization (MALDI)- time-of-flight (TOF)-MS on a Bruker instrument using α-cyano-4-hydroxycinnamic acid as the matrix. The resulting spectra gave lists of masses that were entered into the Profound (NCBI) program. Search criteria were 0.1 Da mass accuracy and a match of at least four tryptic fragments. The ProFound score 'Z-value', indicating the quality of the database search results, had to be higher than 0.5. The SWISS-PROT database (http://www/expasy.ch/sprot/) was used for additional information on pI, MW and amino acid composition of the identified proteins.

3 Results

3.1 Effectiveness of potential therapeutic agents to prevent microvesication

3.1.1 BB 94

In previous experiments, the effective concentration of BB94 in preventing microvesication had been assessed before at 1.0 µg/ml or higher. In addition, it was shown that BB94 can be added as late as 8 h after exposure to HD (Mol, 2003). In the present study, it was investigated whether even longer lag times were allowed between HD exposure and the application of BB 94 to the organ culture medium, without loosing the protective effect of the inhibitor. Therefore, BB94 was added to the organ culture medium in a final concentration of 2.0 µg/ml (4 µM) at 0, 8, 14, 18 and 24 h after the start of the post-exposure incubation. In *Figure 1* it is shown that 2.0 µg/ml BB94 can be added as late as 18 h after exposure to HD, without loosing its inhibiting effect on epidermal-dermal separation.

3.1.2 Furin inhibitor

Furin converts pro-MMP2 and pro-MT1-MMP into their active forms, MMP-2 and MT1-MMP, respectively. Application of a furin inhibitor might therefore reduce or prevent epidermal-dermal separation of skin following HD exposure. The furin inhibitor Dec-RVKR-CMK has been added to culture medium immediately at the start of the post-exposure incubation, in concentrations of 25, 50, 100, 250 or 500 µM. A dose-dependent reduction of microvesication is observed. In the presence of 500 µM furin inhibitor the epidermis and dermis of the skin were not torn apart (*Figure 2*).

Furin inhibitor itself, up to 500 µM was not toxic to the human skin (not shown).

3.1.3 Inhibitor of MMP-1, -3, -7 and -9 (Inhibitor II)

A potent broad range inhibitor of MMP -1, -3, -7 and -9 (N-hydroxy-1,3-di-(4-methoxybenzenesulfonnyl)-5,5-dimethyl-[1,3]-piperazine-2-carboxamide) was added to the post-exposure organ culture medium in concentrations ranging from 0.1 – 20.0 µg/ml. This MMP inhibitor improved epidermal-dermal attachment in skin after HD-exposure, but full protection against HD-induced microvesication was not achieved at the highest concentration tested (*Figure 3B,3C).* The inhibitor itself was not toxic to human skin (not shown).

3.1.4 Inhibitor of MMP-1, -2, -3, -7, -13 (Inhibitor III)

A homophe-hydroxamic acid based broad range inhibitor of MMP -1, -2, -3, -7 and -13 was added to the post-exposure organ culture medium in concentrations ranging from 0.1 – 10.0 µg/ml. A dose-dependent reduction of microvesication was observed and full protection against HD-induced microvesication was obtained with concentrations of 5 µg/ml and higher (*Figure 3D, 3E*). The inhibitor itself was not toxic to human skin (not shown).

3.1.5 MMP2/MMP9 inhibitor

A potent inhibitor of MMP-2 as well as MMP-9 was added to the post-exposure organ culture medium in concentrations ranging from 12.5 – 250 µg/ml. This inhibitor appeared to be toxic to the human skin at concentrations of 25 µg/ml and higher (not shown). At the non-toxic concentration of 12.5 µg/ml the inhibitor was ineffective with regard to reduction of microvesication (*Figure 3F*).

3.1.6 Pancaspase inhibitor

To reduce cell death of keratinocytes, the organ culture medium was supplemented with an inhibitor of all caspase enzymes, Z-FAD-FMK. The inhibitor was added immediately at the start of the post-exposure incubation, in concentrations of 10, 25, 50, 100 or 200 μM. Fully blocked microvesication is observed with 10μM Z-FAD-FMK and higher. In addition, an obvious reduction of necrotic cells is observed (*Figure 4*).

Pancaspase inhibitor itself, up to 200 μM was not toxic to the human skin (not shown).

3.2 Role of laminin-5 in HD-induced microvesication

To investigate whether changes in laminin-5 expression take place during the development of microvesicles, human skin pieces that were exposed for 5 min to HD vapor were examined histologically and immunohistochemically at 4, 8, 16, 24 and 48 h of post-exposure incubation. Histologically, first signs of epidermal cell death are seen at 16 h after exposure, whereas microvesication is overt at 48 h post-exposure (*Figure 5A, 5C, 5E, 5G*). The immunochemical staining pattern for laminin-5 shows that laminin-5 is markedly present as a thick line along the epidermal-dermal junction in control skin (*Figure 5B*). At 16 h following exposure to HD the staining intensity of laminin-5 appears to be reduced and at 48 h the line has become thin and at some places interrupted (*Figure 5D, 5F*). However, when microvesication is blocked by BB94, furin inhibitor or pancaspase inhibitor, the expression of laminin-5 is not improved compared to untreated HD-exposed skin (*Figure 6*). The microvesicles that are seen in the frozen sections that were stained immunohistochemically are artefacts. In histological sections of HD-exposed skin that was treated with these inhibitors no microvesication is observed (*Figures 1, 2, 4).*

3.3 Differential display of HEK proteins associated with exposure to HD

Comparative qualitative analysis of gels obtained at 18, 24, 32 and 42 h of incubation following exposure to 0, 100 or 150 μM HD demonstrated several alterations in protein expression that were associated with exposure to HD. As silver staining is not a quantitative method, only proteins spots that consistently showed a treatment-dependent change in intensity compared to control were referred to as HD-exposure related. No differences were seen between protein patterns obtained from HEK exposed to 100 or to 150 μM HD, neither were differences observed in patterns of cells that were lysed at 24, 32 or 42 h post-exposure (results not shown). Therefore, the silver stained protein expression pattern of HEK exposed to 100 μM HD obtained at 24 h post-exposure is taken as typical. In this protein profile, several spots are observed that are related to HD exposure. Most of them are seen in the area with pI 4.5 – 5.5 and MW 20 -30 kDa. Protein patterns of control and HD-treated HEK at 24 h post-exposure that are found to be typical for this area are shown in *Figure 7.* The protein spots that are indicated with arrows are qualitative treatment-dependent alterations. These difference spots have been subjected to peptide mass fingerprinting resulting in the identification of twelve of them, listed in Table 1. Except for two, the found spots were degradation products of the cytoskeletal keratins 14, 16 and 17 that are present in undifferentiated cultured HEK. Spots **b, c, g, h** and **i** are fragments of type I keratin 14 (K14). Peptide mass fingerprinting of spots **b** and **c** yielded tryptic fragments that matched with amino acid sequences in the C-terminal part of the protein, whereas the tryptic fragments that were observed in spots **g, h** and **i** consisted of amino acid sequences that occur exclusively in the N-terminal part of K14. Peptide mass fingerprinting of spots **a, e** and **f** revealed that they are C– and N-terminal fragments of type I keratin 17 (K17). Spots **d** and **k** were identified as

C- and N-terminal fragments of type I keratin 16 (K16). Finally, spots **l** and **m** have been identified as heat shock protein (HSP) 27.

The presence in the culture medium of the caspase inhibitors Z-FAD-FMK, Z-DEVD-FMK, or Z-VEID-FMK during post-exposure incubation prevented the appearance of the keratin fragments. The HSP27 spots **l** and **m** remain expressed in the presence of caspase inhibitors, indicating that their appearance is independent of caspase activity (*Figure 8*).

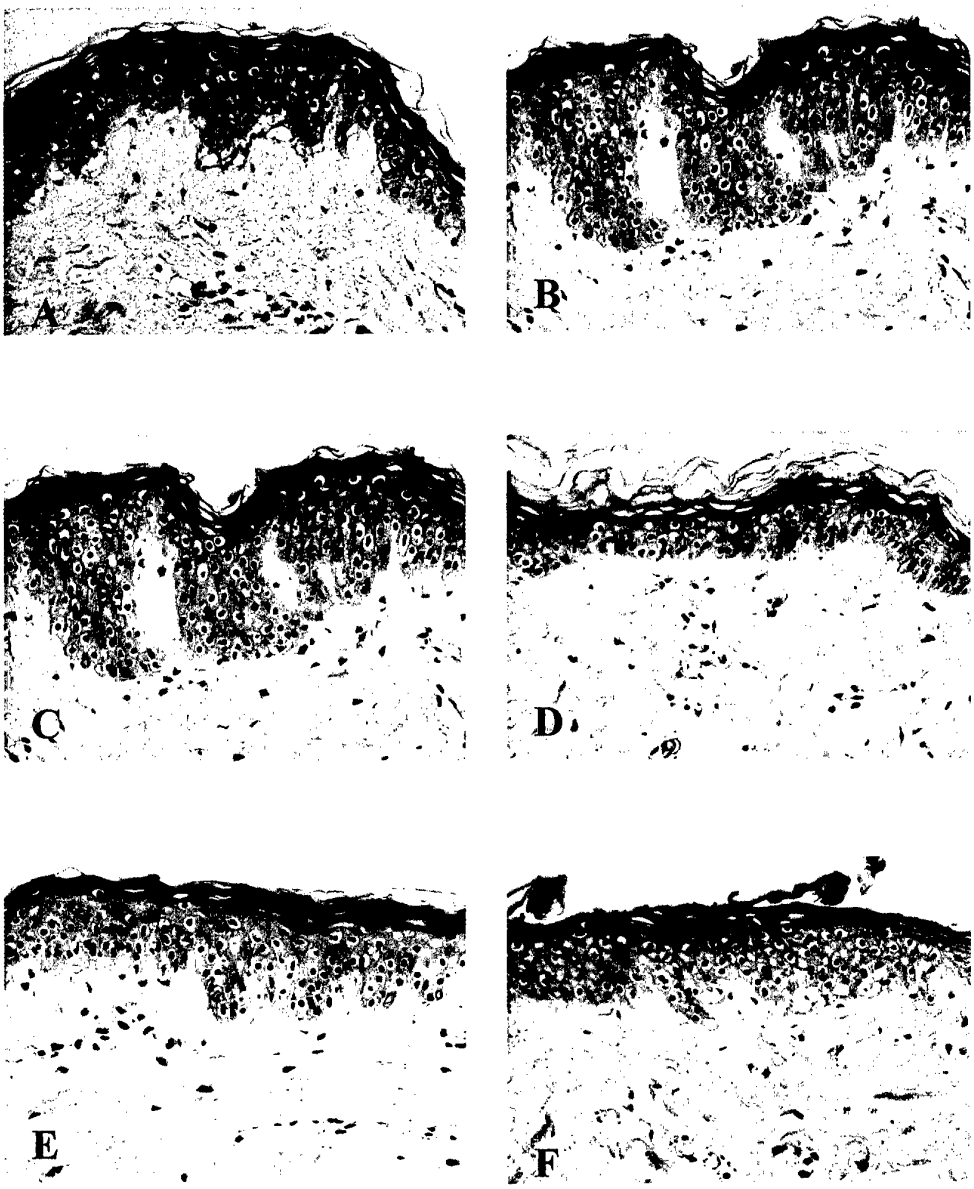


Figure 1. Effects of delayed application of MMP-inhibitor BB94 on microvesication in human skin. Skin has been exposed to saturated vapor of HD for 5 min, and subsequently organ cultured in KBMCa for 48 h. The medium was supplemented with nothing (A) or with 2.0 μg BB94/ml, which was added immediately (B), at 8 (C), 14 (D), 18 (E) or 24 h (F) after exposure. There is still a full protective effect of BB94 on microvesication, when BB94 is added at 18 h after HD exposure.

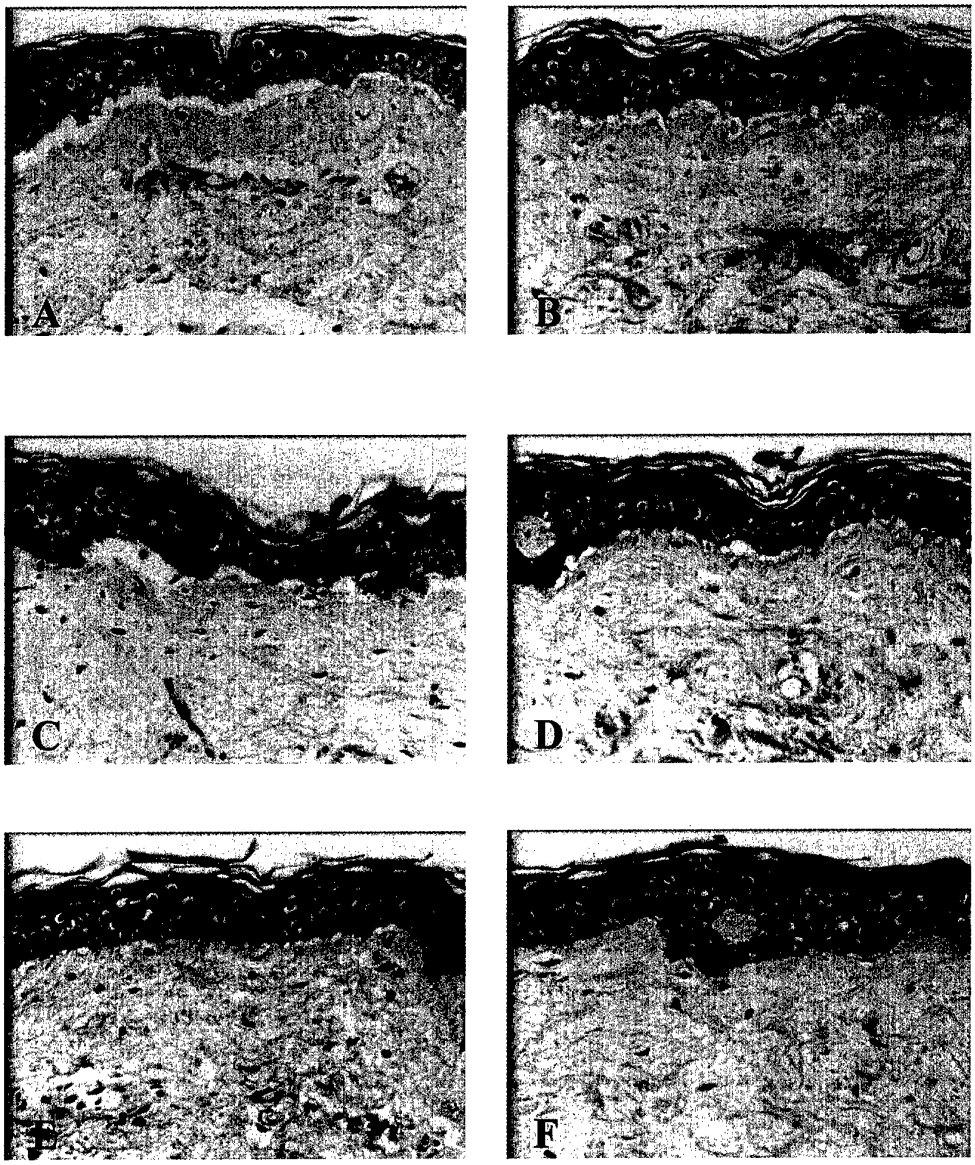


Figure 2. Effects of furin inhibitor Dec-RVKR-CMK on microvesication in human skin that has been exposed to saturated vapor of HD for 5 min. After exposure, skin is organ cultured in KBMCa for 48 h in the presence of 0 (A), 25 (B), 50 (C), 100 (D), 250 (E) or 500 (F) μM furin inhibitor. Microvesication is fully blocked in the presence of 500 μM furin inhibitor.

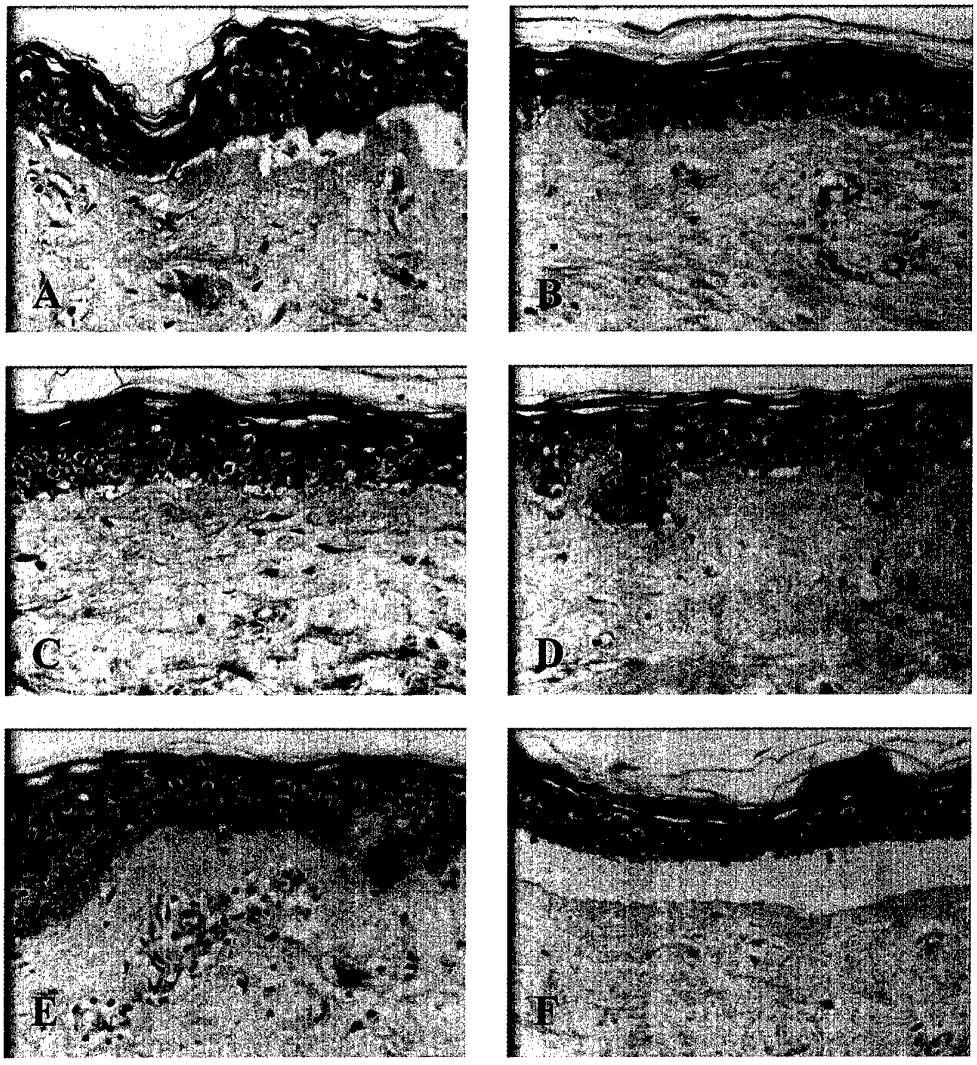


Figure 3. Effects of MMP inhibitor II, MMP inhibitor III and MMP2/MMP9 inhibitor on microvesication in human skin that has been exposed to saturated vapor of HD for 5 min. After exposure, skin is organ cultured in KBMCa for 48 h in the presence of no inhibitor (A); MMP innhitor II 10 μg/ml (B) and 20 μg/ml (C); MMP inhibitor III 2 μg/ml (D) and 5 μg/ml (E); MMP2/MMP9 inhibitor 12.5 μg/ml (F). Microvesication is only fully blocked in the presence of 5 μg/ml MMP inhibitor III.

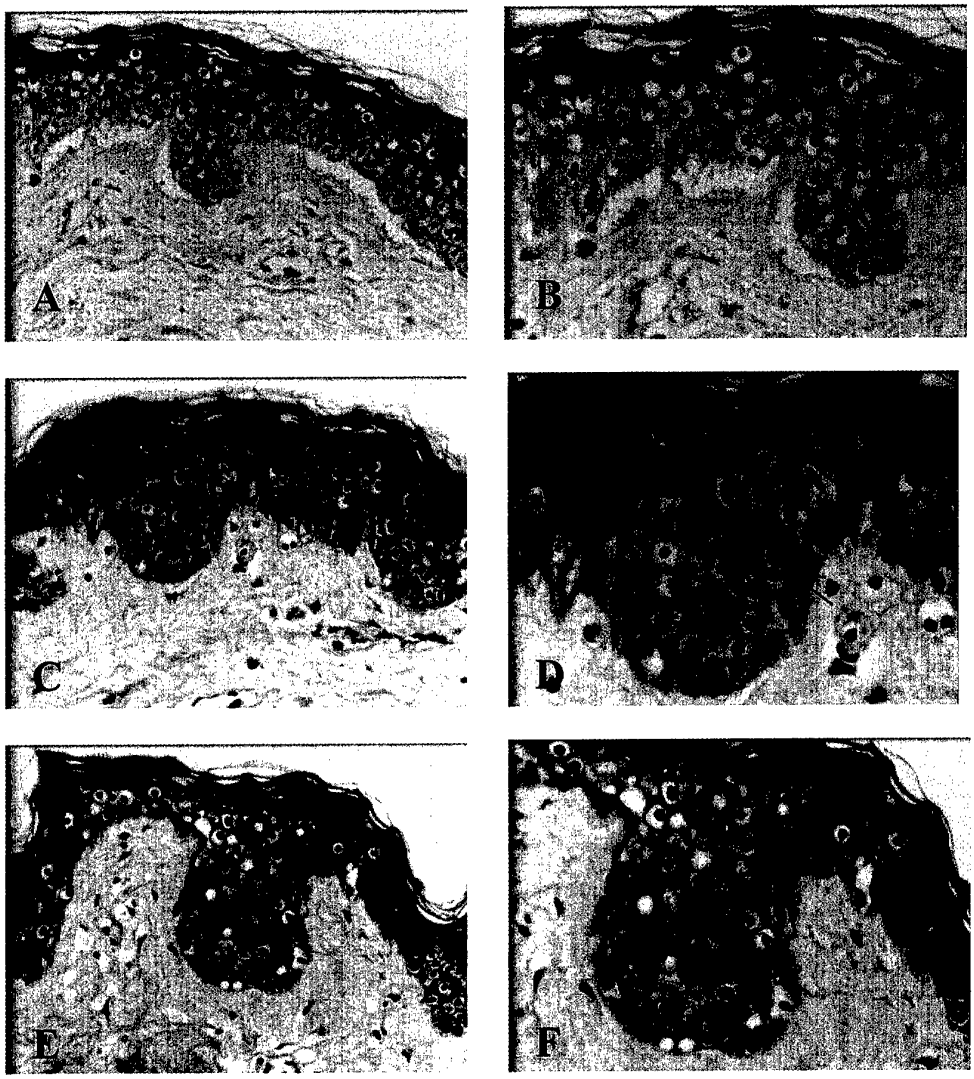


Figure 4. Effects of pancaspase inhibitor on microvesication in human skin that has been exposed to saturated vapor of HD for 5 min. After exposure, skin is organ cultured in KBMCa for 48 h in the presence of no inhibitor (A,B); 10 μM pancaspase inhibitor (C,D); 50μM pancaspase inhibitor (E,F). Pancaspase inhibitor is fully protective at 50μM. Photographs B, D and F are enlargements of A, C and E. Arrows point to typical epidermal cells that maintained a relatively normal appearance.

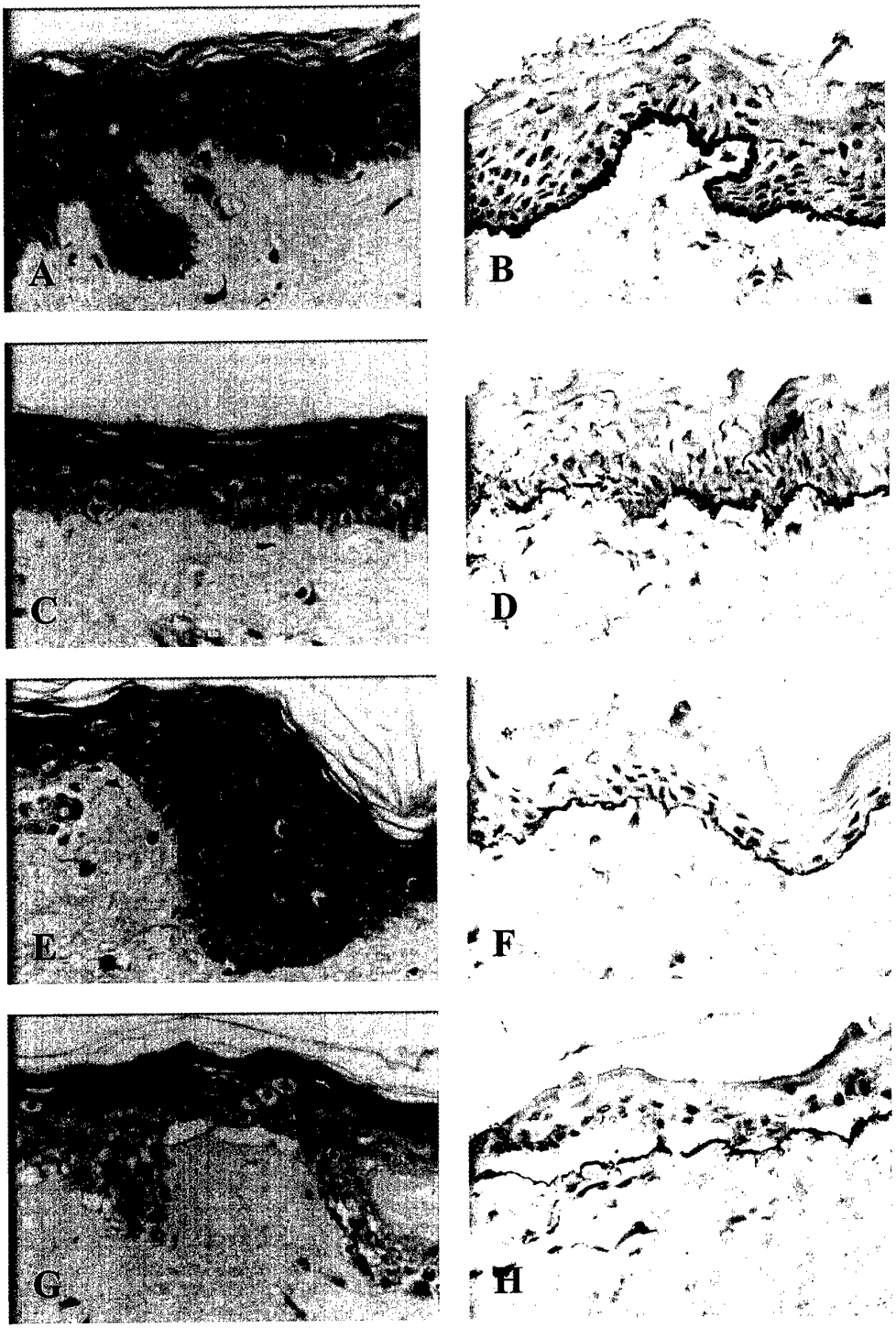


Figure 5. Immunohistochemical staining of laminin-5 in frozen sections of control skin (B) and of HD-exposed human skin at 16 h (D), 24 h (F) and 48 h (H) of post-exposure organ culture. To examine skin morphology during the pathogenesis of microvesication, paraffin sections were stained with hematoxylin/eosin (A, C, E, G). A time-dependent decrease in

staining intensity of laminin-5 along the BM is observed. When epidermis is detached of dermis, laminin-5 remains with the microblister base.

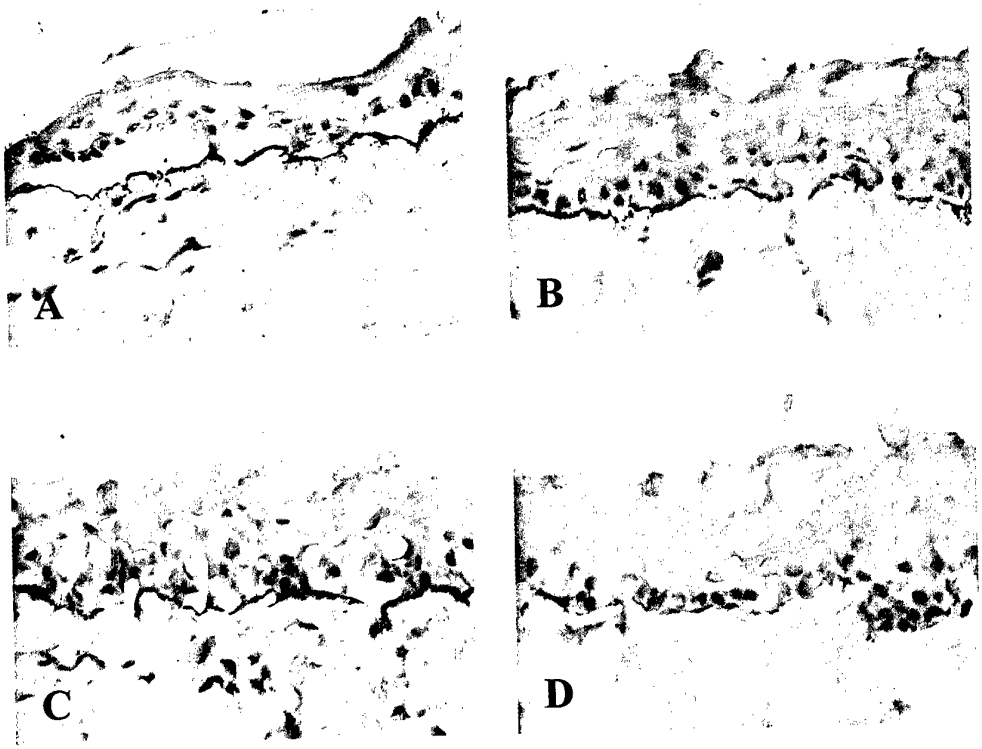


Figure 6. Immunohistochemical staining of laminin-5 in frozen sections of HD-exposed human skin at 48 h of post-exposure organ culture. The medium was supplemented with nothing (A), 2μg/ ml BB94 (B), furin inhibitor Dec-RVKR-CMK (C) or pancaspase inhibitor Z-FAD-FMK (D). The staining intensity of laminin-5 did not change in the presence of each of these inhibitors.

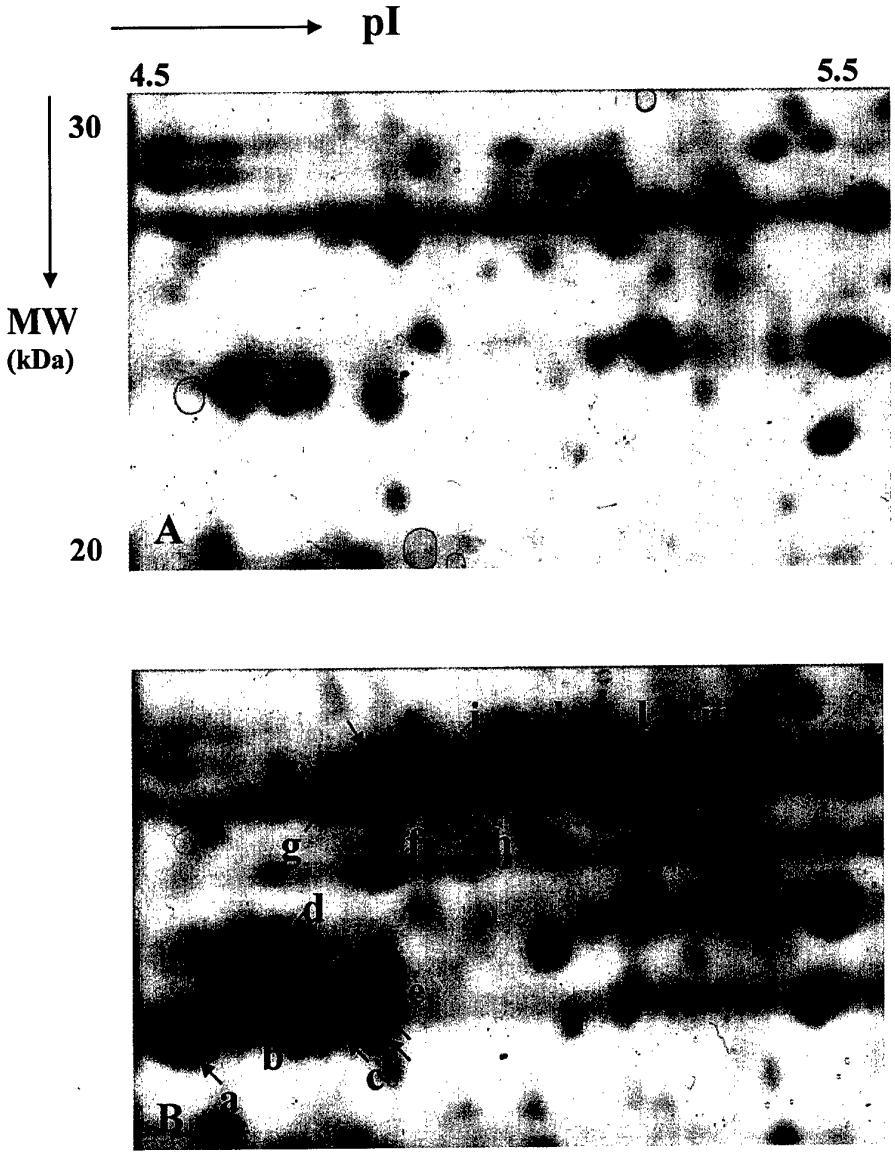
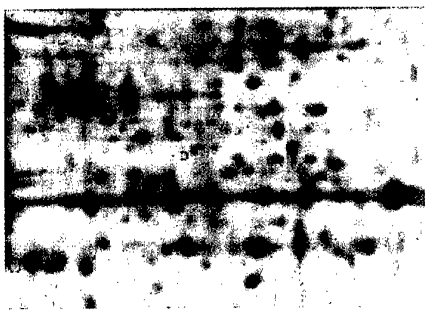


Figure 7. Protein expression in lysates of cultured HEK, collected at 24 h after exposure to 0 (A) or to 100 μM HD. The area shown is confined to pI 4.5-5.5 and MW 20 -30 kDa. The protein spots that are indicated with arrows are qualitative HD-treatment related alterations. Spots annotated with **a** through **m** have been identified and their names are listed in Table 1.

Table 1. List of proteins that were found to be HD-exposure related spots. Proteins were identified by MALDI/TOF/MS analysis. Letters correspond to spots indicated in Figure 7B. Values for MW and pI are according to the SWISSPROT database.

Spot	Protein name	Swiss Prot entry (intact protein)	Theor. MW (kDa)/pI*
a	Keratin 17, C-terminal fragment	Q04695	22.0/5.0
b	Keratin 14, C-terminal fragment	P02533	22.9/5.1
c	Keratin 14, C-terminal fragment	P02533	22.9/5.1
d	Keratin 16, C-terminal fragment	P08779	22.5/4.8
e	Keratin 17, C-terminal fragment	Q04695	22.0/5.0
f	Keratin 17, N-terminal fragment	Q04695	25.9/5.0
g	Keratin 14, N-terminal fragment	P02533	28.5/5.1
h	Keratin 14, N-terminal fragment	P02533	28.5/5.1
i	Keratin 14, N-terminal fragment	P02533	28.5/5.1
k	Keratin 16, N-terminal fragment	P08779	28.6/5.2
l, m	Heat shock protein 27	P04792	22.8/6.0

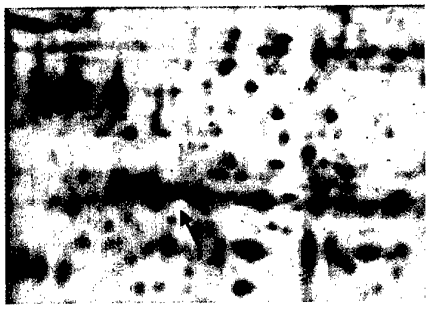


0 μM HD

100 μM HD

100 μM HD + 20 μM pancaspase inhibitor

100 μM HD + 20 μM caspase-3 inhibitor



100 μM HD + 20 μM caspase-6 inhibitor

Figure 8. Effects of various caspase inhibitors on the cleavage of cytoskeletal keratins in HEK that were exposed to 100 μM HD. HEK were incubated with the inhibitors during 3 h pre-exposure and during 24 h post-exposure. In the presence of the caspase inhibitors no keratin fragments were formed. The HD-exposure related expression of HSP27 (red arrows) was not affected by caspase inhibition.

4 Discussion

4.1 Effectiveness of potential therapeutic agents to prevent microvesication

This study showed that HD-induced microvesication in organ cultured human skin can be blocked by the broad spectrum MMP inhibitors BB94 and MMP inhibitor III. Both inhibitors are structurally based on hydroxamic acid, which suggests that also other inhibitors of this class can be used. This finding implicates that MMPs and metalloprotease/disintegrins (ADAMs) like tumor necrosis factor-alpha converting enzyme (TACE), will be the main metalloproteases that are involved in microvesication. In addition, inhibition of furin appeared to be also effective in preventing epidermal-dermal separation after HD-exposure, although a relatively high concentration (500 μM) had to be used to obtain full protection. This proprotein convertase is involved in the maturation of proproteins in the secretory pathway. Processing of MMP-2, MT1-MMP and TACE by furin has been described.

The observation that addition of BB94 as late as 18 h after exposure to HD vapor took place is still effective in blocking microvesication provides evidence that proteolytic degradation becomes critical for epidermal-dermal attachment just after so many hours. Until then, there seems to be a balance between BM protein degradation and synthesis resulting in epidermal-dermal attachment. Based on the finding that at 18 h after HD exposure epidermal cell death becomes substantial, it is hypothesized that the balance then becomes disturbed by failure of *de novo* protein synthesis, whereas protein degradation goes on. The beneficial effect of the pancaspase inhibitor on the histological appearance of the epidermal cells as well as on the epidermal-dermal attachment supports this assumption. The advantageous outcome of inhibition of caspases in HD-exposed skin also points out that apoptosis is at least partially involved in the mechanism of action of HD on the skin. Similar results were obtained from our proteome studies of HEK (section 3.3) and by the group of Rosenthal (Rosenthal et al., 2003). From the results obtained so far, it can be concluded that intervention in the apoptotic proces and in metalloprotease activity are the main targets for therapy against HD-induced injury of the skin.

4.2 Role of laminin-5 in HD-induced microvesication

Laminin-5 is a main adhesion protein of epithelial cells that links hemidesmosomes with the basement membrane. As this heterotrimeric molecule is one of the target proteins that is processed by MMPs, ADAMs and mammalian tolloid metalloproteinase (Pirila et al., 2003; Veitch et al., 2003) the expression of laminin-5 was followed during HD-induced pathogenesis of microvesication. It was observed that when microvesicles are present the expression of laminin-5 along the blister base was reduced and at some sites even absent. The staining of laminin-5 at the dermal side of the blister indicates that the connection with dermal proteins such as collagen VII is still intact, but that the bond with the basal cell integrins has been disrupted. Interestingly, immunostaining of laminin-5 was not improved in skin that was treated with inhibitors of MMPs, furin or caspases, whereas microvesication was blocked. It is concluded that the reduced expression of laminin-5 seems to be not determinative for the development of microvesication. In addition, the results show that the reduced expression of laminin-5 is not caused by enzymes that are inhibited by BB94 and Dec-RVKR-CMK, as the presence of these inhibitors in the organ culture medium following exposure did not affect the decrease in laminin-5 expression.

4.3 Differential display of HEK proteins associated with exposure to HD

The fragments of K14, K16 and K17 are presumably generated by caspase-6 cleavage, based on the presence of the VExD sequence motif in the keratins that is specific for this enzyme (Prasad et al., 1998; Ku and Omary, 2001; Badock et al., 2001). Tryptic digestion of the keratin fragments showed that they contained peptides that were localized either before or after the VExD motif. In addition, the specific caspase-6 inhibitor Z-VEID-FMK completely blocked the appearance of the keratin fragments. The observation that the caspase-3 inhibitor was also effective might mean that activation of caspase –6 occurs downstream of caspase-3. So far, no explanation can be given for the observed isoforms of K14 and K17 fragments with slightly different MWs and pIs.

It was found that in spite of the availability of large amounts of intact K14, K16 and K17 enzymatic fragmentation of the keratins was relatively limited. A possible explanation is that the keratins had become hyperphosphorylated as an early event upon induction of apoptosis. This hyperphosphorylation inhibits caspase cleavage at the VEVD motif of keratin 18 (Ku and Omary, 2001).

It is noted that no fragments were observed of lamins, which are also target proteins of caspase-6 cleavage. Based on their calculated MWs and pIs these fragments should be present in the same area as the keratin fragments. Probably, they are minor spots among the prominent keratin fragments and could not yet be identified.

Apart from the caspase-6 dependent keratin fragments, an acidic isoform of HSP27 has been identified as a HD-exposure related protein. HSP27 is one of several proteins that become phosphorylated by p38 mitogen-activated protein kinase in response to stress (Larsen et al., 1997).

5 Key research accomplishments

- Broad spectrum hydroxamate based inhibitors of MMP, like BB94 and MMP Inhibitor III, fully prevent microvesication in *ex vivo* human skin pieces that were exposed for 5 min to saturated vapor of HD.
- BB94 can be added to the culture medium as late as 18 h after exposure to HD, without loosing its inhibiting effect on epidermal-dermal separation.
- The furin inhibitor Dec-RVKR-CMK blocks HD-induced microvesication in *ex vivo* human skin.
- The pancaspase inhibitor Z-VAD-FMK preserves partially epidermal cell morphology and stops HD-induced microvesication in *ex vivo* human skin.
- Cytokeratins in cultured HEK are fragmented upon exposure to HD at concentrations of 100 and 150 µM and caspase inhibitors prevent this cleavage.
- K14, K16 and K17 are target proteins of caspase-6 cleavage.
- Apoptosis is a key event in the mechanism of action of HD.
- HD-induced microvesication in *ex vivo* human skin is dependent on apoptosis and metalloprotease activity.
- Reduced expression of laminin-5 seems to be not the main cause of microvesication.
- Matrix metalloproteases and related enzymes are not responsible for the reduced expression of laminin-5 during HD-induced pathogenesis of microvesication in *ex vivo* human skin.
- HSP27 becomes phosphorylated in response to HD-exposure.

6 Reportable outcome

M.A.E. Mol and R.M. van den Berg (2003) Inhibitors of matrix metalloproteases block sulfur mustard-induced epidermal-dermal separation. *Poster presentation at 7th Medical Chemical Defense Conference, Munich, Germany*
M.A.E. Mol, R. M. van den Berg, C. van Dijk, and A.L. de Jong (2003) Proteomics as a strategy to study the mechanistic toxicology of sulfur mustard. *Proceedings of the 2003 Meeting of NATO TG004, Medicin Hat, Canada*

7 Conclusions

Microvesication in human skin, due to *ex vivo* exposure to HD, can be blocked by 1) hydroxamate-based inhibitors of MMPs and related enzymes, 2) furin inhibitor and 3) pancaspase inhibitor. These observations give clues to two mechanisms of action to be involved in the development of microvesication: the activity of MMPs and related enzymes and the induction of apoptosis. Treatment of HD-induced skin lesions should be based on intervention in both pathways. From a therapeutic angle, the observation that BB94 can be applied until 18 h after exposure to HD allows non-urgent cure of HD casualties.

During pathogenesis of HD-induced microvesication the expression of laminin-5, one of the key proteins in epidermal-dermal attachment, is reduced. However, MMPs and related enzymes are not responsible for the decrease in laminin-5 expression. Moreover, in the presence of little laminin-5 epidermal-dermal attachment is still possible. Cleavage of other attachment proteins than laminin-5, for example the integrins alpha6beta4 and alpha3 beta1 or collagen XVII (BP230), by MMPs and related enzymes probably causes the break between epidermis and dermis.

The favorable effect of caspase inhibition on maintenance of epidermal-dermal attachment indicated that apoptosis is a key event in the mechanism of action of HD. Impediment of the cell death process appears to be very effective in maintaining epidermal-dermal adhesion. The observation that caspase inhibitors prevent keratin fragmentation in HD-exposed HEK may indicate that an intact cytoskeleton of keratin filaments is essential for epidermal-dermal attachment.

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